

Mechanical robustness of the calcareous tubeworm *Hydroides elegans*: warming mitigates the adverse effects of ocean acidification

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1 ABSTARCT

2 Development of antifouling strategies requires knowledge of how fouling organisms would
3 respond to climate change associated environmental stressors. Here, a calcareous tube built by the
4 tubeworm, *Hydroides elegans*, was used as an example to evaluate the individual and interactive
5 effects of ocean acidification (OA), warming and reduced salinity on mechanical properties of a
6 tube. Tubeworm produce a mechanically weaker tube with less resistance to simulated predator
7 attack under OA (pH 7.8). Warming (29°C) increased tube volume, tube mineral density and
8 tube's resistance to simulated predatory attack. A weakening effect by OA does not make the
9 removal of tubeworms easier except for the earliest stage, in which warming has the least rescuing
10 effect. Reduced salinity (27 psu) did not affect tubes. This study showed that both mechanical
11 analysis and computational modeling can be integrated with biofouling research to provide insights
12 into how fouling communities would develop in future ocean conditions.

13

14 *Key words:* Biofouling; climate change, ocean acidification, global warming, calcification, micro-
15 CT Scanning, finite element analysis, *Hydroides elegans*.

16

17 **Introduction**

18 Carbon dioxide emission by humans is rapidly reducing the global ocean surface pH through ocean
19 acidification (OA) (Caldeira and Wickett 2003, Doney et al. 2009, Zeebe et al. 2008). Calcification
20 by some marine invertebrates can be particularly vulnerable to OA, e.g. they tend to grow slower
21 and produce an impaired /mechanically weaker calcareous structure (Nudelman et al. 2006, Orr et
22 al. 2005, Ries et al. 2009). Calcification products of estuarine and coastal fouling species, such as
23 barnacles and tubeworms, may be severely affected due to multiple anthropogenic (and climate
24 change) threats from OA, warming and reduced salinity (Amaral et al. 2011, Byrne and
25 Przeslawski 2013, Cai et al. 2011, Gibson et al. 2011a, Thomsen et al. 2010). For example, the
26 reduction of carbonate ion concentration with pH during OA caused organisms to produce shells
27 with irregularly arranged minerals at a significantly slower speed (Cohen and Holcomb 2009).
28 Recent studies have shown a declining trend in shell aragonite proportion under OA. This may be
29 due to the preferential dissolution of aragonite over calcite or the favorable production of the more
30 stable yet more brittle calcite over aragonite (Fitzer et al. 2015). The partial loss of aragonite and/or
31 production of disordered crystal morphologies thus resulted in a more brittle shell dominated by
32 heterogeneously sized calcite (Fitzer et al. 2015). Such alternations at nano-scale appear
33 responsible for the production of a shell with lower hardness (Melatunan et al. 2013). Mechanically
34 weaker shells would not only make organisms more vulnerable under predation but also would
35 decrease the intensity of the biofouling community.

36 Under the scenario of multiple and interactive climate change stressors, however, warming
37 tends to accelerate the relative precipitation rate of aragonite over calcite (Burton and Walter 1987)
38 and forces organisms to allocate more metabolic energy for calcification, implying an energy
39 trade-off among shell formation, somatic growth and reproduction (Lannig et al. 2010).

40 Consequently, feeding and metabolic rates increase as compensatory mechanism to maintain and
41 promote growth when conditions are less favorable for calcification such as OA (Melzner et al.
42 2011a). In the absence of adequate food availability or in the absence of physiological plasticity
43 for energy trade-offs, however, OA leads into a delayed calcification and reduced survivorship
44 (Stumpp et al. 2011). A diminished incorporation of organic matrix also occurs to compensate the
45 energetic needs for calcification under stress (Gutowska et al. 2010). Interaction of climate change
46 stressors also plays a vital role in reforming the crystal growth patterns, chemical/elemental
47 composition and the proportion of calcium carbonate polymorph in marine shells (Beniash et al.
48 2010a, Burton and Walter 1987, Chan et al. 2012, Klein et al. 1996). Despite the complex interplay
49 between environment and biofouling species (McDonald et al. 2009), our understanding of the
50 integrative effects of multiple climate-change-related environmental stressors on the mechanical
51 behaviors of the exoskeletons of biofouling species is still in its infancy (Poloczanska and Butler
52 2010).

53 Among various biofouling species, the serpulid polychaete *Hydroides elegans* is recognized as
54 an ecologically and economically important species in warmer coastal oceans (Nedved and
55 Hadfield 2009). This serpulid worm builds, through complex and controlled calcification process,
56 a calcareous tube to protect itself from predation and/or environmental threats (Tanur et al. 2009,
57 Vinn and Kupriyanova 2011, Vinn et al. 2008). Accumulation of these calcareous tubes on man-
58 made structures including ship hulls and cooling water pipelines poses a serious challenge for
59 fouling control (Qian and Pechenik 1998, Qian et al. 2007). A series of recent studies have shown
60 that the calcified tubes of *H. elegans* is disorganized with altered chemical composition and
61 reduced mechanical strength under OA (Chan et al. 2012, Lane et al. 2013). Interestingly, elevation

62 of seawater temperature from the optimal 24°C to 29°C level tends to rescue these worms from the
63 above mentioned OA effects (Chan et al. 2013b).

64 These observations raises a question: Are climate change related stressors in the coastal oceans
65 is more harmful for calcareous skeletal (or shell or tube) formation in combination than alone?
66 Here, micro-computed tomography (micro-CT) and scanning electron microscopy (SEM) methods
67 were used to examine the effect of multiple climate change stressors on the topography of the
68 calcareous tube surface of the tubeworm. Micro-force testing was used to assess the mechanical
69 resistance of the tubes to external loading. Finite-element analysis (FEA) was carried to simulate
70 the mechanical performance of different structured tubes under predator attack. Such a quantitative
71 relationship analysis between structural polymorphs and tube mechanical performance will
72 provide new understanding of the mechanisms by which tube-forming worms will cope with near
73 future climate change scenarios.

74 **Materials and Methods**

76 **Test animal**

77 Mature seruplid tubeworms, *Hydroides elegans*, were collected from a floating fish farm located
78 in Yung Shue O, Hong Kong (22°27'N, 114°23'W), in April 2012. The surface seawater
79 temperature, pH and salinity at the time of collection were ~23 °C, ~8.1 and ~34 psu, respectively.
80 In the laboratory, eggs and sperm collected from more than 100 individuals were mixed for
81 fertilization in filtered seawater (FSW, 0.22 µm) at ambient temperature, pH and salinity condition.
82 In *H. elegans*, normally >80% matured eggs get fertilized within few minutes success rate.
83 Fertilization success rate was not significantly affected by either low salinity at 27 psu or elevated
84 temperature at 29°C or decreased pH at 7.8 (Ackley Lane, personal communication). Therefore, in

85 this study, fertilization was carried out under ambient control seawater temperature, salinity and
86 pH condition. Fertilized embryos were used in experiments.

87 **Multiple climate change stressors: experimental design**

88 An orthogonal factorial experimental design was used to study the individual and the interactive
89 effects of pH, salinity and temperature on the *H. elegans* calcareous tubes mechanical properties.

90 In this study, seawater pH was used as a proxy to measure the ocean acidification intensity. Two
91 pH values were employed: pH 8.1 (ambient) and pH 7.8. Similarly, two temperatures (23°C =
92 ambient; and 29°C) and salinity (34 psu = ambient; 27 psu) were used. There were 8 treatment
93 combinations with 4 replicates. The treatments are abbreviated as follows: ambient or control (C),
94 3 individual treatments (reduced salinity = S; decreased pH = P; elevated temperature or warming
95 = T), 3 two-stressor-combined treatments (reduced salinity and decreased pH = SP; elevated
96 temperature and reduced salinity = TS; elevated temperature and decreased pH = TP) and a three-
97 stressor-combined treatment (elevated temperature, reduced salinity and decreased pH = TSP).
98 Description of this experimental design and treatment manipulation methodology were explained
99 in details in our previous paper (Chan et al. 2012, Chan et al. 2013b).

100 Treatment levels were selected based on both current seasonal variation in the *H. elegans*
101 habitat and projected global environmental (climate) change scenario for the year 2100 (Pechenik
102 et al. 2007, Zeebe et al. 2008). Seasonal fluctuations of seawater temperature, salinity, pH and
103 dissolved oxygen levels in the adult collection site (Yung Shue O, Hong Kong) were shown in
104 Figure S1. The monthly average salinity was reduced from ~ 34 psu to ~27 psu during the rainy
105 season, i.e. June to August. During the peak summer (July and August), the surface temperature
106 was elevated from ~ 15°C to ~29°C. The surface seawater pH fluctuated within a narrow range of
107 7.8 to 8.2 (NBS scale). Unlike temperature and salinity, pH did not fluctuate with season in the *H.*

108 *elegans* habitat (Fig. S1). Due to regional and global environmental and climate change drivers,
109 the surface seawater temperature is estimated to increase by 3 to 4°C within this century, while
110 surface average pH is projected to decrease by 0.3 units (i.e. from 8.1 to 7.8) within 100 years
111 (Zeebe et al. 2008). In addition, the average surface salinity in several coastal habitats of South
112 China is expected to be steadily reducing (from 34 psu to 27 psu) due to climate change associated
113 heavy precipitation (Deng et al. 2014).

114 Temperature was elevated and maintained using immersion heaters and circulating water bath.
115 Salinity was lowered to 27 psu by diluting natural seawater with distilled water. The ambient pH
116 8.1 was reduced to 7.8 through directly injecting CO₂ enriched air into culture tanks. About 1000
117 ppm of CO₂ in ambient air was required to decrease pH 8.1 to the treatment level of 7.8. The 100%
118 CO₂ gas was constantly mixed with air to a required level using a variable area flow meter
119 controller (Cole-Parmer Inc.) Because reduction of seawater pH due to CO₂ addition is dependent
120 on temperature and salinity treatments, CO₂ concentration in the bubbled air was maintained at a
121 constant level of 1000 ppm for all decreased pH treatments such as P, TP, SP and TSP. The CO₂
122 concentration in the inflowing air was frequently measured using Quantek Model 906 Carbon
123 Dioxide Analyzer (Quantek Instruments, Inc., USA). Carbonate system parameters such as
124 saturation level of aragonite and pCO₂ were calculated using the CO2SYS program with the
125 equilibrium constants K₁, K₂ and K_{SO₄} (Table S1). Temperature and pH were monitored daily
126 using electrodes (SG2, Mettler-Toledo, Hong Kong), which were calibrated using NBS/NIST
127 standards (at pH 4, pH 7 and pH 10). Total alkalinity (TA) was measured using Alkalinity Titrator
128 (AC-A2, Apollo SciTech's Inc, USA) using poisoned (10 µl of 250 mM mercuric chloride) samples
129 (50 mL) and certified seawater reference material (Batch 103, A.G. Dickson, Scripps Institution
130 of Oceanography).

131 Under these 8 treatments, embryos of *H. elegans* (with density of 10 ml^{-1}) were cultured to 18
132 day old adults according to the standard procedure (Chan et al. 2013a) using a plastic culture tanks
133 with 1 L capacity After the exposure period, adult tubeworm samples were collected and rinsed
134 with MilliQ water to remove salts. Cleaned tubes were preserved in 70% ethanol (Clode et al.
135 2011). Tubes were dried at room temperature prior to the following measurements.

136 **Tube morphology and size measurement**

137 The tube morphology was examined using resin-embedded tubes with a scanning electron
138 microscope (SEM) (Vinn et al. 2008). The hardened resin was sectioned using an ultramicrotome
139 (Ultracut S, Leica, Germany), glass knife and diamond saw (DiATOME Ultra 45°). Sectioned
140 samples were soaked in 0.5 M EDTA for etching (Tanur et al. 2010). Samples were mounted onto
141 aluminum stubs using adhesive carbon tape and the cross-sectioned side of the tube was mounted
142 facing up. Silver paint was applied to surround the resin surface and provide better connection to
143 the aluminum stub in order to minimize electron charging, specimens were coated with 50 nm
144 thick gold – palladium alloy. Tube morphology and ultrastructure was examined by observing the
145 samples at an accelerating voltage of 5 kV using a LEO 1530 Gemini FSEM (Zeiss, Germany).
146 The average tube thickness and circular radius were measured using Image J (ver. 1.46r, NIH).

147 **Tube density, volume and 3D surface topography measurements**

148 Tubes properties such as volume and density were obtained digitally by 3D reconstruction and
149 scanning with micro-computed tomography (micro-CT) scanning system (SkyScan 1076,
150 Belgium). The system was mounted by a 3×10^{-6} cubic mm voxel size with spatial resolution of 15
151 μm . Prior to the scanning, tubes attached on polystyrene petri dishes were dislodged manually into
152 small pieces (e.g. 4-10 tubes per replicate), transferred into a micro-CT chamber for X-ray
153 scanning. After a universal scanning signal threshold was established, the tube volume data was

154 quantified by calculating the number of observable pixels of each tube after accounting for a
155 background signal. Tube density data were obtained through relative comparison with two
156 phantoms used for bone density standard (Celenk and Celenk 2012). About 76-345 X-ray images
157 were generated per specimen for each 3D model (SkyScan, Belgium) and reconstruction was done
158 using software CTvol (v 2.2.1.0). Two to four individual were visualized as 3D reconstructions in
159 each replicate.

160 **Crushing force measurement**

161 The crushing force, which is defined as the compressive force required crushing a tube, was used
162 to indirectly measure the resistance power of the tube to external mechanical loading. In this study,
163 the applied external mechanical loading mimicked an attack by the predators such as fishes and
164 crabs. Measurements of the crushing force were carried out with a microforce testing system
165 (Tytron 250, MTS System Co., USA). Prior to testing, intact tubes (4-10 tubes per replicate) that
166 were used for micro-CT scanning were mounted on the stationary platform of the microforce
167 testing apparatus. A metal plane (diameter of 1.5 cm) was mounted on the movable clamp to exert
168 compression on the tube. A force-displacement curve was generated for each specimen at a loading
169 rate of 0.05 mm/sec. The crushing force was recognized as the “yielding” point on the curve (Hill
170 1952). The resistance power of tubes obtained from all the eight treatments treatment conditions
171 were measured using 2-4 tube samples per replicate cultures.

172 **Finite-element modeling**

173 Finite element analysis (FEA) (ABAQUS, Dassault Systems, France) was employed to simulate
174 the mechanical behaviors of tubes with different structures as a response to different environmental
175 conditions. In our FEA, the tubes were modeled as semi-cylindrical shells with cross-sectional
176 geometries such as diameter and wall thickness obtained from the measurements of SEM images.

177 Our attention was mainly focused on the tubes from the control (C) and elevated temperature (T)
178 treatment because the latter treatment resulted in a unique morphological features on the tubes
179 including a pair of longitudinal keels along both sides and enlarged basal region of the tube, as
180 illustrated in Fig. 1. It was assumed that the basal region of the tube was firmly attached to the
181 substrate and that the tube was purely elastic with Young's modulus and Poisson ratio being 50
182 GPa and 0.3, respectively. A rigid flat plane was compressed onto the tubes to simulate predator
183 attack. 8-node quadratic plane strain element (CPS8 in ABAQUS) was adopted in our FEA.

184 **Data analysis**

185 The interactive effects of decreased pH, reduced salinity and elevated temperature on tube volume,
186 tube density, crushing force were tested by a three-way ANOVA. Density data failed to satisfy the
187 assumption of variance homogeneity, they were rank-transformed before running a three-way
188 ANOVA. The statistical power of a three-way ANOVA is generally low to rule out treatment
189 effects due to random sample variability (Moran 2003). In this case, Student's t-test was applied
190 to study the separate treatment effects on tube thickness, tube circular radius and fracture toughness.
191 The t-test for equal variance was used when $p > 0.05$ while t-test for unequal variance was used
192 when $p < 0.05$.

193 **Results**

194 **Analysis of the system of carbonate chemistry**

195 Throughout the experiments, the pH, salinity and temperature value were maintained and met the
196 expectation of experimental values. In addition, the fluctuations of all corresponding variables
197 were acceptable and consistent among replicate in any treatment condition (Table S1). The
198 saturation state of aragonite (Ω_A) was larger than 1.0 in all treatments. The decreased pH treatment
199 alone led to Ω_A of 1.3, and the combined effect of decreased pH and reduced salinity lowered the

200 Ω_A to 1.0. Elevated temperature combined with either decreased pH or reduced salinity elevated
201 the Ω_A to 1.6 while under combined effect of 3 stressors (elevated temperature, reduced salinity
202 and decreased pH), the Ω_A was 1.2. Temperature effect alone raised the Ω_A significantly from
203 ambient level (control) of 2 to 2.6.

204 **Morphological features and size parameters**

205 The calcareous tubes built by the tubeworm were strongly influenced by environmentally and
206 climatically relevant levels of pH, temperature and salinity treatments (Figs. 1a-h). The cross-
207 sectional view of the tube demonstrated that the tubeworms produced a pair of keels along the
208 longitudinal axis at an elevated temperature (T, TP, TS and TSP) (Hedley 1958), as indicated in
209 dotted-line circle as longitudinal keels (Figs. 1 b, d, f and h: white dashed circles). At all four
210 treatments with elevated temperatures, the basal regions in contact with the substrate showed
211 considerable enlargement (Figs. 1 b, d, f and h), they were wider when compared to the ambient
212 temperature treatments (Fig. 1a, c, e and g). Tube thickness and radius were significantly reduced
213 by decreased pH at both temperatures (C > P; T > TP), these effects appeared to be alleviated by
214 an increase in temperature (T) (Fig. S2; Table S2). The positive effect of temperature was very
215 prominent in that all the tubes at higher temperatures were thicker and bigger than the control
216 (T/TS/TP/TSP > C). There was no significant impact by the level of salinity reduction employed
217 in the current study and three factors (T, S and P) showed no significant interaction (Table 1).

218 **Volume and spatial density distribution of the tubes**

219 3D reconstruction of the tubes incubated at different treatment conditions was conducted using
220 micro-CT scanning (Figs. 2a-c). The two sides and the posterior end were displayed as “pores” or
221 “disconnections” in the image (Fig. 2a; P, SP, TP, TSP) because the density there is lower than the
222 universal detectable threshold value. These tubeworms built their tubes with lower density

223 materials during their early juvenile stages. Furthermore, the density distribution of the tubes was
224 represented in a color map (Fig. 2b, 3D model; white = higher density; red = lower density). The
225 tubes show a higher density at the anterior opening than that at the posterior tail. The inner layer
226 was denser than the outer layers as shown in cross-sectional distribution (Fig. 2c; longitudinal
227 section). A density colour map was generated from average density ($n = 4-6$ per replicate) to
228 display a spatial distribution of tube density along the normalized length (0 = posterior tail; 1=
229 anterior opening) of the longitudinal axis of the tubes under different treatment conditions (Fig.
230 3a). As shown in the density map in Fig. 2c and 3c, anterior ends of the tubes were generally denser
231 than posterior region. When density of tube is analyzed with the normalized length, the initial
232 density when tubes were formed in the earliest time (near 0-0.3, posterior region) experienced
233 similar density reduction when treated with decreased pH. The effects of different treatment
234 conditions on tube density (Fig. 3b) and volume (Fig. 3c) suggest that elevated temperature (T)
235 gradually mitigates the detrimental effect of P and enhances density and volume (Figs. 3b and c;
236 Tables 1 and S3). The tube volume almost doubled under elevated T, even at both TP and TSP.
237 Reduced salinity, however, did not influence the tube density or volume.

238 **Mechanical resistance of tubes to external crushing force**

239 The crushing force measured using micro-force testing facility. The tube mechanical resistance to
240 external loading was significantly decreased by pH at both temperatures ($C > P$; $S > SP$; $T > TP$;
241 $TS > TSP$) (Fig. 4; Table 1). On the other hand, tubes developed from an elevated temperature
242 required a significantly higher crushing force when compared to control and all other treatments
243 (Fig. 4; Tables 1 and S4; $T > C$; $TS > S$; $TP > P$; $TSP > SP$). When compared to the control, the
244 mechanical performance of shells produced at higher temperature despite the negative impact of
245 reduced pH may be as strong as or stronger than in the control (Fig. 4; $C \leq TSP/TP/TS$).

246 **Mechanical resistance of tubes against simulated predator attack**

247 Tubes incubated in the higher temperature treatments exhibit morphological features (Fig. 1) such
248 as (1) a pair of longitudinal keels and (2) enlarged basal contact. To shed light on the structural
249 importance of these morphological features, finite element analysis (FEA) was applied to compare
250 the mechanical behaviors of tubes of different structures subjected to a simulated predator attack.
251 Firstly, we compared the response of the tube incubated in elevated temperature (Fig. 5b; elevated
252 temperature treatments; T) with that of the control (Fig. 5a; Control; C). The averaged geometric
253 parameters obtained from SEM images were applied in the construction of these models
254 (thickness: C = 17 μm ; T = 40 μm ; radius: C = 110 μm ; T = 206 μm). Under the same applied load
255 of 35 N, the maximum principal stress (MPS) developed in both structures are shown in Fig.5a
256 and b respectively. Clearly, in the C model, high stress concentration can be observed at the side
257 and in the vicinity of the base, while in the T model, no clear stress concentration is observed. The
258 greatest values of the MPS in both cases are 12.1 GPa, and 5.3 GPa, respectively, implying that
259 the C model is much easier to be fractured in comparison with the T model given that their
260 materials' properties are the same. One may ascribe such advantage of the T model in mechanical
261 behavior to its larger cross-sectional area compared to the C model. To eliminate this factor, a
262 scaled-up C model (SC) was built up by magnifying the C model to such a degree that the cross-
263 sectional area is equal to that of the T model. Similar FEA was conducted on the SC model. Fig.
264 5c shows the MPS field developed in the SC model under the same load. It can be seen that stress
265 concentrations still exist even though the greatest value of the MPS is reduced to 5.3 GPa, which
266 is still ~ 8.7 times larger than that in the T model. Such enhancement of mechanical performance
267 exhibited by the T model can be attributed to its unique structures including the paired longitudinal
268 keels and the enlarged base.

269 The evolutions of the local MPS peaks at apical, lateral and basal positions with the
270 compression load for tubes incubated under different treatment conditions (C - black, T - blue and
271 SC - red) were summarized in Figure 5d. As expected, during the whole loading process, the T
272 model exhibits much lower MPS compared to the C and SC models, implying a much higher
273 resistance to the external compression.

274 **Discussion**

275 This study determined the effects of multiple environmental variables as predicted in climate
276 change scenarios on the morphology, volume, density and mechanical properties of the biofouling
277 tubeworm, *Hydroides elegans*. Reduction in pH by ocean acidification (OA) as projected within
278 this century, i.e. pH 7.8, would lead to smaller, thinner, less dense and weaker tubes. Reduction in
279 spatial density distribution within the tube structures upon OA treatment suggests the presence of
280 corrosive action of decreased pH. The question remains whether an interacting climate change
281 variable such as warming and reduced salinity could worsen the negative effects of OA.
282 Interestingly, warming counteracts the detrimental effect of OA. Simulated predator attack using
283 computer modeling approach suggests that the tubeworms are capable of making specific
284 structural arrangement at elevated temperature environment to strengthen the mechanical weak
285 points, i.e. by having a pair of longitudinal keels and enlarged basal regions. Together with
286 enhanced tube growth in the warmer environment, the tubeworms are expected to build a
287 mechanically stronger tube under projected near-future climate change. Reduced salinity at the
288 tested level of 27 psu did not influence the mechanical properties of the tubes.

289 ***Effect of decreased pH***

290 Under projected near-future pH 7.8, the tubeworms produce a mechanically weaker tube (with
291 smaller size, density and volume) with less resistance to simulated predator attack, posing a

292 possible mechanical challenge for their survival in the future coastal ocean. In line with our
293 observation, previous studies reported that a reduction in seawater pH would lead to smaller tube
294 size associated with impaired ultrastructure, altered mineral composition, less hardness and weaker
295 capacity to resist predator attack in *H. elegans* (Chan et al. 2012, Li et al. 2014). As a bimineralic
296 calcifier, compositional alterations towards higher calcite to aragonite ratio occurs in response to
297 decreased pH due to a preferential dissolution of the more soluble aragonite (Beniash et al. 2010b,
298 Ries 2011). Such alteration of mineral composition could lead to a more compromised mechanical
299 performance with more brittle calcite content (Chan et al. 2013a, Weiner and Addadi 1997). In
300 accordance with a previous study, the middle layer of the tube was generally denser and harder
301 when compared to outer and inner layers of the tube (Li et al. 2014). Direct exposure of the
302 outermost and the innermost parts of the tube to seawater with a decreased pH may further
303 differentiate the layer structures of the tube and have an impact on the mechanical performance
304 (Melzner et al. 2011b, Tanur et al. 2010, Vinn 2011, Vinn 2013).

305 In this study, the pH reduction did not lead to an under-saturation of aragonite and calcite and
306 therefore direct dissolution of calcified structures would be expected to be unlikely. However, a
307 seawater environment with a decreased pH level of 7.8 can be unfavorable for calcification, due
308 to the accumulation of protons as the inhibitory by-product (Jokiel 2013). This is because an
309 environment that is high in protons may slow down the ion influx and efflux of the organelles
310 responsible for maintaining a cellular environment for shell deposition (Dickinson et al. 2012,
311 Digby 1968, Mount et al. 2004). Furthermore, a decreased pH environment can cause hypercapnic
312 suppression of metabolism that impede calcification (Miles et al. 2007, Todgham and Hofmann
313 2009). Decreased pH symptoms such as impaired ultrastructure (Hahn et al. 2011, Hahn et al.
314 2012), altered crystal alignment (Fitzer et al. 2014, Wolfe et al. 2013), and increased amorphous

315 calcium carbonate content (Chan et al. 2012) may have also contributed to the observed low
316 density tubes with a mechanically weaker resistance to predator attack under decreased pH. Similar
317 impairments during calcification under decreased pH have also been found in several calcifying
318 marine invertebrates such as bryozoans (Lombardi et al. 2011, Rodolfo-Metalpa et al. 2010), corals
319 (Cohen and Holcomb 2009), sea urchins (Byrne et al. 2011), oysters and mussels (Beniash et al.
320 2010b). However, the degree of impairment appeared to be taxonomically variable and dependent
321 on the intensity of pH reduction (Ries et al. 2009).

322 *Effect of warming*

323 Warming triggered a production of mechanically stronger tube with higher resistance to externally
324 simulated predator attack. Similar results were found in our previous study that used
325 nanoindentation tests to determine the mechanical properties of the *H. elegans* tube (Chan et al.
326 2013a). There are several possible explanations for such a positive effect of moderate warming.
327 Presumably, additional metabolic energy gained through increased metabolism at elevated
328 temperature may have been allocated to calcification or tube building. For example, oysters are
329 able to produce thicker shells under warming (Ruddy et al. 1975). Additionally, seawater
330 temperature commonly correlates with the higher likelihood of amorphous calcium carbonate
331 (ACC) stabilization and Mg incorporation into the lattice structures of CaCO_3 (Chan et al. 2013a);
332 an increase in crystallized ACC (Addadi et al. 2003, Weiner and Addadi 1997) and Mg/Ca-rich
333 structures has also been shown to enhance mechanical resistance of shells (Beniash et al. 1997,
334 Gong et al. 2012, Ma et al. 2007). Moreover, the observed positive effect of warming on tube
335 mechanical resistance may be mediated by the alteration of mineral crystal organization rather than
336 by the increase in tube thickness as previously thought. Enhanced metabolism and energy
337 availability at warmer condition could have induced the tubeworms to allocate energy for the

338 production of protective outer organic layer over the tubes (Vinn 2011, Vinn 2013). The presence
339 of such an outer layer reported to protect tubes and shells in the corrosive decreased pH
340 environment (Tunncliffe et al. 2009, Vinn 2011, Vinn 2013), has been considered to be limited in
341 the serpulid tubeworms (Stanley 2006). A moderate increase in production of organic covers may
342 be a valuable strategy to escape the damage of ocean acidification.

343 In contrast, warming reduced the mechanical strength of the bivalve (*Mytilus edulis*) without
344 altering its shell morphology and mineral composition but through reducing shell surface area
345 (Fitzer et al. 2015, Mackenzie et al. 2014). Warming also altered molluscan shell microstructure,
346 thickness and width of its aragonite tablets in nacre (Füllenbach et al. 2014). These bivalves are
347 generally forced to produce impaired shells with less surface area due to re-allocation of energy
348 from shell formation to temperature-related increases in maintenance costs (Gibson et al. 2011b,
349 Mackenzie et al. 2014). In our study, however, the *ad libitum* fed tubeworms prevented potential
350 energy shortage so the animals were able to produce a mechanically stronger tube with additional
351 structures under highly energy demanding warmer condition.

352 ***Multiple stressors effect***

353 Although *H. elegans* produced mechanically weaker tubes at decreased pH (P), a combination of
354 elevated temperature (T) and P at TP treatment induced the production of a mechanically stronger
355 tube to withstand external force or simulated predator attack. Similar phenomenon of rescuing
356 effects by warming has been reported in sea urchins (Brennand et al. 2010, Byrne et al. 2010) and
357 corals (McCulloch et al. 2012) when their upper thermal tolerance was not breached. Interestingly,
358 a similar restoration effect in shell strength by warming was also found in predatory snails (Landes
359 and Zimmer 2012). While the mechanisms behind this rescuing effect remain obscure, and the
360 overall community response is still uncertain, under a warmer environment, animals could have

361 an elevated metabolism that provides the much needed energy resources to override the negative
362 effect of a decreased pH (Lannig et al. 2010, Pörtner et al. 2005). Nevertheless, the interactive
363 effect of these two stressors appears to be highly species specific. For example, warming tends to
364 exacerbate the negative effects of pH reduction in crabs (Hoffmann and Parsons 1989), abalone
365 (Byrne et al. 2010) and oysters (Talmage and Gobler 2011).

366 The posterior part of the tube is generally less dense than the newly produced anterior part.
367 Warming appeared to only enhance the anterior part of the tube that is produced at a later stage of
368 juvenile development and calcification. After larval metamorphosis, the juvenile tube of *H.*
369 *elegans* demonstrate a succession in tube mineral composition with increasing calcite/aragonite
370 ratio and reducing ACC content with age (Chan et al. 2015). Therefore, the difference in tube
371 properties along the longitudinal axis could be due to differences associated with different rate of
372 development. Alternatively, the posterior end is more susceptible to dissolution, with higher ACC
373 and aragonite content, may also lead to more loss in shell materials and compromised mechanical
374 properties (Andersson et al. 2008, Beniash et al. 1997).

375 In marine invertebrates, decreased pH with or without other environmental stressors generally
376 initiate a metabolic depression (Pörtner et al. 2005). On the other hand, acclimation at elevated
377 temperature lead to an increase in aerobic metabolism (Pörtner 2008). However, elevated
378 temperatures, beyond tolerance limits cause negative effects on physiology and leads to an
379 irreversible physiological breakdown. For *H. elegans*, 29°C is within their upper thermal tolerance
380 limit (Qiu and Qian 1997). However, temperature beyond this critical level might reduce their
381 thermal window and aerobic scope, especially under the interaction of multiple stressors. Since
382 elevated temperature or decreased pH effects are mediated through metabolic alterations and
383 availability of food in the environment (Melzner et al. 2011a). Although *H. elegans* performance

384 beyond 29°C or below to their lower temperature tolerance limit at 15°C under decreased pH or
385 reduced salinity was not tested in *H. elegans*, fitness of several marine invertebrates has been
386 shown to be negatively affected due to physiological tradeoffs at thermal limits under decreased
387 pH or reduced salinity (Metzger et al. 2007). Therefore, the observed rescuing capacity of warming
388 in *H. elegans* is highly dependent on elevated temperature level, thermal window and the
389 availability of food in the environment.

390 *Finite element analysis (FEA)*

391 Calcification occurs in a variety of structural and mechanical forms (Holcomb et al. 2014). Such a
392 diversity accounts for specialized structural and mechanical properties of marine invertebrates.
393 Some of these features are crucial in the face of global environmental change, however, there are
394 not many ways to describe or quantify changes in such properties in calcifying organism. This
395 study employed FEA modeling analysis (Hamm et al. 2003, He et al. 2013, Rayfield 2007) to
396 demonstrate the significance of structural features in coping with environmental stress. We
397 demonstrate that a minor increase in shell material allocated to the suitable structures, i.e. basal
398 tube region and a pair longitudinal keels, can lead to a 8.7 times enhancement in mechanical
399 resistance to simulated predator attack. The application of FEA modeling utilizes databases of
400 conventional measurements, such as tube radius and thickness and density data, which are
401 commonly available in the current literature. FEA allows the quantification of and comparison
402 between calcifiers with different taxonomic groups, providing valuable comparison of resistance
403 to predator attack, hence potential community shift in future environment. Thus, FEA offers a
404 quantitative method for better evaluating animal's structural design, relationships between
405 predator and prey, as well as minimizing disputable virtual-age effect though eliminating age-
406 difference in size and mechanical properties (Martin et al. 2011).

407 ***Implications on fouling community growth and control***

408 The effects of climate change on biofouling species and its community are of growing concern
409 (Poloczanska and Butler 2010). According to our results, development of calcareous biogenic
410 fouling materials by the tubeworms are strongly influenced by projected near future levels of pH
411 and temperature in the environment, yet biofouling research had not incorporated these climate
412 change variables while testing the efficacy of various fouling control strategies. Our research
413 suggests that the biofouling strength of the tubeworm is likely to be enhanced by warming in the
414 future ocean, and more effective antifouling or removal method may be necessary. As the primary
415 biofouling organism, (Nedved and Hadfield 2009), increase in tube resistance to predator attack
416 would simultaneously facilitate the build-up of fouling community such as barnacles, mussels and
417 bryozoan. Results of this study provide new insights into how various marine calcifiers respond to
418 multi-factor climate change during their early life. We also provided a generalizable method to
419 study the mechanical performance of different biofoulers, which would identify the stronger
420 calcifiers in the biofouling community and provide a clearer antifouling objective.

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Table 1. Summary of F-ratios obtained from the results of three-way ANOVA analysis of the individual and the interactive effects of decreased pH (P), reduced salinity (S) and elevated temperature (T) on calcareous tube size and mechanical properties of *Hydroides elegans*. Significant effects ($p < 0.05$) are bolded. Treatment abbreviations: C: Control; S: reduced salinity (27 psu); P: decreased pH (pH 7.8); T: elevated temperature (29 °C); SP: reduced salinity and decreased pH (27 psu; pH 7.8); TS: elevated temperature and reduced salinity (29 °C; 27 psu); TP: elevated temperature and decreased pH (29 °C; pH 7.8); TSP: elevated temperature, reduced salinity and decreased pH (29 °C; 27 psu; pH 7.8).

*Density data were rank-transformed due to inhomogeneous variance

Size parameters	S	P	T	SP	ST	PT	SPT
Tube thickness	0.136	49.376	196.336	5.339	0.007	2.814	1.136
Tube radius	1.042	57.085	533.357	0.370	4.185	1.483	0.041
Tube volume	1.759	10.044	93.160	1.046	1.071	1.047	0.663
Mechanical properties							
Tube density	0.015	19.568	35.989	0.092	0.092	0.015	0.367
Crushing force	0.902	22.066	43.279	0.232	0.008	1.248	0.031

Figure Legends

Figure 1 (a-h). Representative scanning electron micrographs of cross-sectional area of *Hydroïdes elegans* cultured under the individual (T, S and P) and interactive effects (SP, TS, TP and TSP) of decreased pH (P), reduced salinity (S) and elevated temperature (T). a: C: Control; b: T: elevated temperature (29 °C); c: S: reduced salinity (27 psu); d: TS: elevated temperature and reduced salinity (27 psu; pH 7.8); e: P: decreased pH (pH 7.8); f: TP: elevated temperature and decreased pH (29 °C; pH 7.8); g: SP: reduced salinity and decreased pH (27 psu; pH 7.8); h: TSP: elevated temperature, reduced salinity and decreased pH (29 °C; 27 psu; pH 7.8). Circle in dotted line indicates the present of a pair of longitudinal keels in the tubes treated with elevated temperature (T, TS, TP and TSP).

Figure 2 (a-c). 3D reconstruction models of the calcareous tubes of *Hydroïdes elegans* obtained from micro-computed tomography (micro-CT) images analysis. Models are shown for the tubes obtained from eight treatments. (a) Original 3D scanning images of the tubes, (b) density distributional map of the 3D scanned images, and (c) density distribution along the longitudinal section. Letters representing different treatments are shown on the bottom row. Treatment abbreviations: C: Control; S: reduced salinity (27 psu); P: decreased pH (pH 7.8); T: elevated temperature (29 °C); SP: reduced salinity and decreased pH (27 psu; pH 7.8); TS: elevated temperature and reduced salinity (29 °C; 27 psu); TP: elevated temperature and decreased pH (29 °C; pH 7.8); TSP: elevated temperature, reduced salinity and decreased pH (29 °C; 27 psu; pH 7.8).

Figure 3 (a-c). The tube density and volume obtained from micro-computed tomography (micro-CT) images of *Hydroides elegans* to individual and interactive effects of decreased pH (P), reduced salinity (S) and elevated temperature (T). (a) A heat map summary the effect of eight water treatments on a spatial distribution of tube density along the normalized tube length (where 0 = anterior opening; 1 = posterior end, average value from 4-6 samples). (b) Effect of treatments on tube density. (c) Effect of treatments on tube volume. Treatment abbreviations: C: Control; S: reduced salinity (27 psu); P: decreased pH (pH 7.8); T: elevated temperature (29 °C); SP: reduced salinity and decreased pH (27 psu; pH 7.8); TS: elevated temperature and reduced salinity (29 °C; 27 psu); TP: elevated temperature and decreased pH (29 °C; pH 7.8); TSP: elevated temperature, reduced salinity and decreased pH (29 °C; 27 psu; pH 7.8). Error bars represent the mean \pm SD of four replicates.

Figure 4. The tube mechanical resistance to external crushing force of *Hydroides elegans* to individual and interactive effects of decreased pH (P), reduced salinity (S) and elevated temperature (T). Treatment abbreviations are as shown in Figure 1. Error bars represent the mean \pm SD of four replicates.

Figure 5 (a-d). Finite-element analysis of the mechanical resistance of *Hydroides elegans* tube against simulated predator attack. The calculated maximum principal stress (MPS) field in different structured tubes under the same compression (35 N/m) of a rigid plane: (a) Control (C) model simulating the typical tube of tubeworms incubated in control environmental condition; (b) T model simulating the typical tube of tubeworms incubated in elevated temperature treatment condition; (c) scaled-up control model (SC) with the same cross-sectional area of the T model; (d)

Evolutions of the local peaks of MPS at apical, lateral, and basal regions with the compression load.